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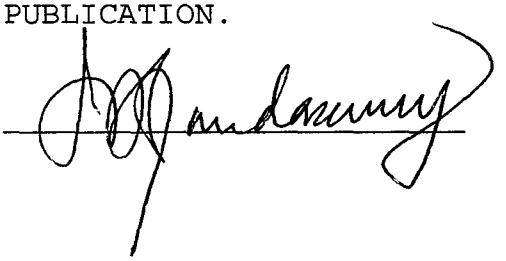

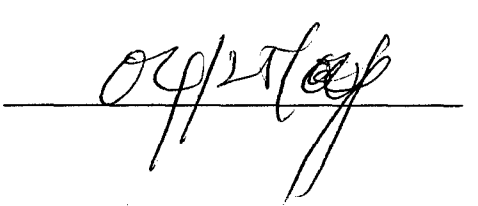

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13. ABSTRACT ***(Maximum 200 Words)***

We identified LKB1, a Ser/Thr kinase in which mutations are responsible for Peutz-Jegher disease, is a substrate of caspase-8 in vitro. Peutz-Jegher syndrome is a rare autosomal dominant disease characterized by a predisposition to develop benign and malignant tumors of many tissue origins including breast cancers. We discovered that LKB1 is a novel pro-apoptotic protein that mediates cell death in a p53-dependent manner. Furthermore, we showed that the endogenous LKB1 protein is recruited to mitochondria upon induction of apoptosis. In normal small intestine, we found that the expression levels of LKB1 are higher in the tip of villus and highly upregulated in the cytoplasm of isolated epithelial cells with pyknotic nuclei. In contrast, the small intestine of Peutz-Jegher patients showed mosaic expression of LKB1 with much of the polyps lacking LKB1 staining. Consistent with its role in regulation of apoptosis, we found a dramatic reduction of apoptotic cells in polyps of PJS patients. We propose that inhibition of apoptosis is one of the key factors responsible for the formation of multiple benign intestinal polyps in PJS and possibly for the development of malignant tumors as well.

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(4) Introduction.

In our previous report, we showed that we have successfully developed an in vitro screening system for identification of caspase substrates. We characterized one of the substrates that we identified, Bid, to be an important intracellular signal transducer from Fas receptor on the cytoplasmic membrane to mitochondria. This paper (Li et al. Cell. 1998. 94, 491-501.), which was published in Cell, was recently cited as one of the two most cited papers published in the last two years (The Scientist. 8/2000). The first author of that paper, Dr. Honglin Li, has since moved on to his own laboratory at the Northwestern University and has taken the Bid project with him. The works in my lab have been concentrated on some of the other substrates that we have identified in the caspase substrate screen. In this report, I describe our recent work on LKB1, a Ser/Thr kinase in which mutations have been shown to be responsible for Peutz-Jegher disease. LKB1 was also identified as a caspase-8 substrate in vitro in our screen. Peutz-Jegher syndrome is an autosomal dominant disease. Early manifestations suffered by PJS patients include melanocytic macules of the lips and multiple hamartomatous polyps in the gastrointestinal tract[1,2]. Later in life PJS patients have a dramatically increased incidence of cancers that originate from a wide variety of tissues [3,4]. The genetic locus responsible for the majority of PJS cases has been mapped to chromosome 19p13.3 [5,6] and the PJS gene has recently been cloned and found to encode a Ser/Thr protein kinase named LKB1/STK11 [6,7].

Disruption in the homeostatic balance between cell proliferation and cell death is a key to cancer development [13]. Tumor cells must not only proliferate without regard to environmental clues, but also evade cellular apoptosis sensor mechanisms. The cellular tumor suppressor gene p53 plays a prominent role in this process because of its potent ability to inhibit cell proliferation as well as to activate apoptosis [14]. Genotoxic stimuli activate p53 by rapidly increasing both p53 protein levels and its transcriptional activation efficacy. Once activated, p53 either causes cell growth arrest or apoptosis. It has been shown that p53 plays a major role in modulating apoptosis in tumor cells by responding to signals such as ionizing radiation, chemotherapy and hypoxia [10]. The importance of p53 as a tumor suppressor is evidenced by the frequent loss of p53 in tumors and the aggressive growth that these tumors display. As expected, tumors missing p53 have a low number of apoptotic cells[15]. While it is clear that p53 plays a critical role in regulating apoptosis, the mechanism by which p53 induces apoptosis, however, is controversial and complicated by its apparent ability to induce apoptosis through transcriptional dependent and transcriptional independent mechanisms [16-22]. It has been reported that p53 activity induces the expression of Bax [20], a pro-apoptotic member of the Bcl-2 family, and genes involved in regulating cellular redox balances[22]; both of which may contribute to p53 mediated apoptosis.

Here we demonstrate that the Peutz-Jegher Syndrome gene product, LKB1, may be one of the key signal transduction molecules in p53-dependent apoptosis. We propose that a deficiency in one of the pathways leading to p53-dependent intestinal epithelial cell apoptosis may be a primary cause for benign hamartoma formation in PJS patients, and may render tissues highly susceptible to further molecular perturbations that lead to malignant transformation.

(5) **Body.**

Induction of apoptosis by LKB1

To examine the possible role of LKB1 in apoptosis, we transfected the GFP-tagged wild type LKB1 into HT1080 fibrosarcoma cells, which have wild type p53[23]. The expression of LKB1 induced cell death (Figure 1A). Flag-tagged wild type LKB1 induced apoptosis to a similar extent (data not shown). LKB1 consists of a N-terminal kinase domain and a putative regulatory domain in its C-terminus. To determine if the C-terminal domain of LKB1 negatively regulates LKB1 activity, we constructed an expression vector for the kinase domain alone (a.a.1 to 309) (KDA). Transfection of the KDA expression vector into HT1080 cells induced apoptosis significantly more efficiently than wild type LKB1 (Figure 1A & B), suggesting that the C-terminal domain plays a role in negatively regulating LKB1 apoptotic activity. Next, we overexpressed two constructs likely to be deficient in kinase activity; an invariant nucleotide binding site mutant K78M LKB1 and a derivative construct missing the first 88 amino acids (Δ88 LKB1). Both of these mutants do not cause an appreciable increase of cell death as compared to control (Figure 1B), suggesting that the kinase activity of LKB1 is needed for the induction of apoptosis by LKB1. A western blot analysis confirmed that the apoptosis induced by LKB1 expression constructs and the protection against apoptosis conferred by zVAD and Bcl-x_L (see below) are not due to differences in the levels of protein expression (Figure 1C).

To determine whether caspases are involved in LKB1 induced apoptosis transfected cells were treated with the caspase inhibitor z-VAD-fmk. As shown in Figure 1B, LKB1 induced cell death was effectively blocked in z-VAD-fmk treated cells indicating that caspases are critical for mediating the downstream apoptosis pathway induced by LKB1. To determine whether caspase-3-like downstream caspases are activated in cells expressing LKB1, we immunostained cells with CM1, an antibody that recognizes only the activated form of caspase-3 [24]. We found that cells expressing LKB1 KDA are positive for CM1 staining and furthermore, that this staining is blocked by z-VAD treatment or the co-expression of Bcl-xL (Figure 1D). These results indicate that LKB1 expression induces the activation of caspase-3-like downstream caspases. Interestingly, a significant reduction in cell death was observed when LKB1 KDA were co-transfected with either Bcl-xL or a caspase-9 dominant negative [25] (Figure 1A & B). This protection suggests that the mitochondrial pathway of apoptosis may be involved in LKB1-mediated cell death. To directly test this possibility, the release of cytochrome c from mitochondria in response to expression of LKB1 was determined. HT1080 cells were transfected with GFP-KDA and immunostained for cytochrome c in the presence of zVAD to prevent nuclear apoptosis. We found that in cells that express LKB1 KDA, the staining of cytochrome c is greatly diminished and dispersed throughout the cell (compare transfected versus non-transfected cells). This suggests that LKB1 causes the release of cytochrome c from mitochondria (Figure 1E, a-f). Although the presence of zVAD prevents the disintegration of nuclei, it does not block the release of cytochrome c from mitochondria (Figure 1E, a-c). As a control, overexpression of K78M LKB1 did not induce the release of cytochrome c (Figure 1E, g-i). Based on these data, we conclude that the mitochondrial pathway of apoptosis, including cytochrome c release

and activation of caspase-9 and caspase-3, is likely to be involved in the cell death mediated by LKB1.

Identification of an activating mutant and dominant negative mutants of LKB1

To characterize the LKB1 kinase activity and its phosphorylation pattern, we transfected HT1080 cells with wild type and derivative constructs of LKB1. Expressed proteins were immunoprecipitated and incubated in an *in vitro* kinase assay. We found that the wild type LKB1 was heavily phosphorylated whereas the phosphorylation of K78M LKB1 was greatly reduced, suggesting that LKB1 undergoes autophosphorylation (Figure 2A). In Xenopus, the Thr-197 residue of XEEK1 has been shown to be autophosphorylated,[10]. To test whether the equivalent residue in mammalian LKB1, Thr-189, is phosphorylated [6] we mutated Thr-189 to Ala-189. The phosphorylation of T189A LKB1 mutant was also significantly reduced when compared to the wild type (Figure 2A). These data suggest that like XEEK1, LKB1 is phosphorylated at Thr-189. This phosphorylation is most likely due to autophosphorylation by LKB1, although we have not ruled out the possible involvement of a coimmunoprecipitating kinase. In either case, the regulatory domain appears to be required for the phosphorylation as the KDA protein is not phosphorylated (Figure 2A). In addition, LKB1 kinase activity is also needed for phosphorylation, as the K78M LKB1 protein was not phosphorylated to any significant amount (Figure 2A).

To determine the functional role of Thr-189 phosphorylation, we transfected T189A into HT1080 cells. Interestingly, we found that T189A LKB1 mutant is much more active in inducing apoptosis than LKB1 in HT1080 cells (Figure 2B). These data suggest that phosphorylation of Thr-189 negatively regulates LKB1 activity.

Since K78M LKB1 does not induce apoptosis, we tested if it could function as a dominant negative of LKB1. Co-transfecting the constitutively active T189A LKB1 with K78M LKB1 significantly reduced the apoptosis induced by T189A LKB1. In contrast, K78M LKB1 had no effect on KDA activity (Figure 2B), suggesting that K78M LKB1 can function as a dominant negative mutant and that this activity relies on the presence of the regulatory domain.

To determine if certain rare LKB1 point mutations identified in Peutz-Jegher patients function as dominant negative mutants, we generated a human LKB1 K78I expressing construct mimicking a mutation found in a Peutz-Jegher patient [12]. Transfection of human wild type LKB1 induces apoptosis of HT1080 cells at levels similar to the wild type mouse LKB1 (Figure 2C). Transfection of K78I LKB1 did not induce apoptosis; furthermore, cotransfection of K78I LKB1 with wild type human LKB1 significantly inhibited apoptosis induced by wild type human LKB1 (Figure 2C). These results suggest that the Peutz-Jegher mutation K78I LKB1 functions as a dominant negative mutant.

Induction of apoptosis by LKB1 is p53-dependent

p53 is proposed to play a major role in mediating apoptosis of tumor cells [26]. Therefore, we asked if LKB1 induced apoptosis requires wild type p53. We transfected wild type EF cells with LKB1 expression constructs and found that wild type LKB1 and KDA both induce apoptosis with equal efficiency (Figure 3A). This is in contrast to HT1080 cells where the LKB1 KDA induced apoptosis more efficiently than that of wild type LKB1. Since HT1080 cells are of fibrosarcoma origin, these results suggest that

HT1080 cells may express an inhibitor of LKB1, which may not be present in wild type EF cells.

In contrast to that of wild type EF cells, the wild type LKB1 or the LKB1 KDA are completely incapable of inducing apoptosis of p53-/- EF cells [27] (Figure 3A). To ask if the inability of LKB1 to induce apoptosis in p53-/- EF cells could be complemented by wild type p53, LKB1 was co-transfected into p53-/- EF cells with an expression construct of wild type p53. We found that the expression of wild type p53 partially but significantly complemented p53-/- EF cells for LKB1 induced apoptosis (Figure 3A). These results suggest that p53 is required for LKB1 induced apoptosis.

Since Bax has been proposed to be one of the downstream mediators of the p53 apoptosis pathway, we tested if LKB1 induced apoptosis requires Bax. We transfected Bax-/- EF cells [28] with LKB1 and its derivative constructs. Unlike p53-/- EF cells, Bax-/- EF cells remain sensitive to LKB1 and KDA induced apoptosis. Thus, we conclude that p53 is essential for LKB1 induced apoptosis but this function does not depend on Bax.

To test whether there is a general requirement for p53 in LKB1 induced apoptosis, we inactivated p53 in HT1080 cells by infecting them with retrovirus containing a dominant negative p53 (p53.175H) [29]. Control virus or dominant negative p53 mutant virus infected HT1080 cells were transfected with LKB1 or KDA expression constructs and cell death was measured (Figure 3B). The ability of p53.175H to function as a dominant-negative was confirmed by showing that p21 expression is inhibited (see later, Figure 7A). We found that while control virus infected cells were still sensitive to apoptosis induced by LKB1, cells expressing the dominant negative p53 acquired resistance to LKB1 (Figure 3B). Taken together, we conclude that there may be a general requirement for wild type p53 function in LKB1 induced apoptosis.

Mitochondrial localization of LKB1 during apoptosis is p53-dependent

The mitochondrion is an important intracellular amplifier of apoptotic signals [30]. Many types of apoptotic signals converge upon the mitochondria and cause the release of cytochrome c into the cytoplasm, which in turn activates caspase-9 via forming complex with Apaf-1 [25]. In a majority of HT1080 cells transfected with the LKB1 expression construct, overexpressed LKB1 is evenly distributed throughout the cytoplasm and nucleus (Figure 4A, top panel). In about half of the HT1080 cells transfected with KDA construct and in a smaller proportion of HT1080 cells transfected with LKB1 construct, however, we observed that GFP-positive aggregates form around the nuclei and co-localize with the mitochondrial-specific dye Mitotracker (Figure 4A, top and middle panels). Thus, overexpressed LKB1 kinase domain alone and to a less extent, the full length LKB1 are recruited to mitochondria. The efficiency of mitochondrial recruitment correlates with the apoptosis inducing ability of LKB1 and KDA (P. Karuman and J. Yuan, unpublished observations). The mitochondrial recruitment is evident before the appearance of nuclear condensation and thus, appears to precede the onset of morphological cell death (Figure 4A. See below). To determine if the kinase activity is required for mitochondrial recruitment of LKB1, we transfected HT1080 cells with the kinase dead mutant K78M LKB1. In contrast to LKB1 and the KDA, the K78M LKB1 is localized in both cytoplasm and nucleus but not in mitochondria. Thus, the kinase activity of LKB1 is required for LKB1 recruitment to the mitochondria.

To determine if mitochondrial translocation of LKB1 is a cause or consequence of apoptosis, we cotransfected HT1080 cells with KDA and Bcl-xL. Coexpression of Bcl-xL strongly inhibited apoptosis induced by KDA (Figure 1A and B); co-expression of Bcl-xL, however, had no effect on mitochondrial recruitment of the LKB1 kinase domain alone (Figure 4. The bottom panel). This suggests that the mitochondrial recruitment of LKB1, is not a consequence of apoptosis.

Since LKB1 induced apoptosis requires p53, we asked if the mitochondrial recruitment of LKB1 requires p53. The subcellular localization of over-expressed LKB1 kinase domain in control and p53 dominant negative virus infected HT1080 cells was determined. Interestingly, we found that the LKB1 kinase domain was not recruited to the mitochondria in HT1080 cells expressing p53 dominant negative mutant (Figure 5A and 5B), suggesting that the wild type p53 activity is needed for KDA translocation. To further confirm the role of p53 in mediating LKB1 translocation, we transfected wild type and p53-/- EF cells with the KDA construct. In wild type EF cells, KDA was found frequently in the mitochondria (Figure 5C); however, in p53-/- EF cells KDA was mostly found in the cytoplasm and nucleus (Figure 5D). These results suggest that p53 may regulate an activity required for the mitochondrial localization of LKB1. This is in striking contrast with the inhibition by Bcl-xL: Bcl-xL has no effect on translocation of LKB1 to the mitochondria but inhibits a downstream step, most likely one that involves the prevention of mitochondrial damage induced by LKB1.

To determine the subcellular localization of endogenous LKB1, we generated an anti-LKB1 antibody that specifically recognizes the 55kD LKB1 protein on western blot (Figure 6A). Analysis of the subcellular distribution of LKB1 by immunostaining revealed that endogenous LKB1 was present in both the cytoplasm and the nucleus (Figure 6B). To determine if the endogenous LKB1 translocates to mitochondria upon induction of apoptosis, we stained HT1080 cells that were treated with Fas or paclitaxel. In both paclitaxel and Fas treated HT1080 cells, a significant portion of LKB1 was found in the mitochondria (Figure 6C & D). As cytoplasmic but not nuclear LKB1 signal diminished in apoptotic cells, we conclude that the cytoplasmic LKB1 is recruited into mitochondria. The translocation of LKB1 to the mitochondria is an early event in apoptosis, because a significant portion of cells with mitochondrial localized LKB1 still exhibit normal nuclear and cell morphology (Figure 6C & D). Thus, translocation of LKB1 to the mitochondria probably represents a critical early step in LKB1 induced apoptosis.

Specific Inhibition of apoptosis induced by microtubule disruption by dominant negative K78M LKB1

To determine if LKB1 is required for p53 dependent apoptosis, we infected HT1080 cells with retrovirus expressing K78M LKB1, p53.175H or control virus. Cells were selected using antibiotics carried by the vectors for 4-6 days and their sensitivity to different apoptosis inducers was determined. The function of p53 dominant negative mutant was examined by checking the expression of p53 and p21 in p53 mutant virus infected cells (Figure 7A, a & b). The expression of p21 was significantly inhibited in p53 dominant negative mutant expressing cells (Figure 7, Aa). Western blot analysis showed that cells infected with K78M LKB1 virus expresses the flag-tagged LKB1 mutant protein (Figure 7A, d). Control and HT1080 cells expressing K78M LKB1 or p53 175H were treated with different doses of 5-fluorouracil, doxorubicin, paclitaxel or vincristine and apoptosis was measured 24 hrs later using MTT assay. The cells

expressing p53 175H exhibited considerable resistance to apoptosis induced by all four agents in the doses tested (Figure 7B). Interestingly, the cells expressing K78M LKB1 showed resistance only to paclitaxel and vincristine, the two agents that disrupt microtubule dynamics, but not to that of 5-fluorouracil or doxorubicin (Figure 7B). In addition, the LKB1 K78M expressing cells are also not resistant to Fas induced apoptosis (data not shown). These results suggest that LKB1 is not required for all p53-dependent apoptosis or for Fas induced apoptosis, but rather, is specifically involved in p53-dependent apoptosis following microtubule disruption.

The expression patterns of LKB1 in normal small intestine and Peutz-Jegher polyps

To examine the expression of LKB1 protein, we immunostained small intestinal samples from normal adult and Peutz-Jegher patient biopsies (4 each) with anti-LKB1. LKB1 is expressed in intestinal epithelial cells in a gradient fashion so that the highest level is at the villus tip (Figure 8Aa & b). While LKB1 expression is detected in nuclei of epithelial cells, the cytoplasm of epithelial cells around the villus tip is highly positive for LKB1 immunostaining (Figure 8Aa & b). Adult small intestinal epithelial cells, which are generated through division of the multipotent stem cells that are found anchored near the base of crypts, migrate over time as a vertical coherent bands from the crypt, up the villus to the villus tip where they die [31]. Thus, the spatial relationship of epithelial cells along the villus also reflects their age: the cells closer to the villus tip are older than those distal to it. Accordingly, LKB1 expression is higher in older cells than younger cells. Therefore, our data suggests that the gradual upregulation of LKB1 expression during epithelial cell aging, may be responsible for their death and extrusion at the villus tip.

In addition to the gradual upregulation of LKB1 expression along the villus, we also observed individual dying cells with pyknotic nuclei exhibiting very high levels of LKB1 staining in the cytoplasm (Figure 8Ac & d). This again suggests that upregulation of LKB1 expression is an important control for its activation.

To determine the expression of LKB1 in Peutz-Jegher patients, we immunostained polypectomy samples from Peutz-Jegher patients with anti-LKB1. The normal LKB1 expression gradient is found in the areas of normal intestinal epithelium adjacent to or in between polyps (Figure 8Ba). The staining is specific for the anti-LKB1 antibody, as no staining was observed when the primary antibody was omitted (Figure 8Bb). LKB1 immunostaining in Peutz-Jegher polyps is consistently and significantly weaker than that of normal biopsy control small intestinal samples; and much of the samples completely lacks LKB1 immunostaining (Figure 8Bc & d). These results are consistent with the finding that most of Peutz-Jegher mutations are complete loss-of-function alleles [6,7,11,12]. Small patches of weak LKB1 staining can be found in certain areas of Peutz-Jegher polyps (data not shown), suggesting that certain epithelial cells in polyps may not have lost both LKB1 alleles. Our data are in agreement with a previous proposal that the intestinal tissues of PJS patients are mosaic and that the polyps arise in areas that have lost the wild type copy of LKB1 gene [5].

Lack of epithelial apoptosis in small intestinal polyps of Peutz-Jegher patients

A prediction that may be drawn from a functional role of LKB1 in regulating apoptosis is that intestinal polyposis in PJS patients may be caused by a deficiency in apoptosis. To test this hypothesis directly, we examined biopsy and polypectomy samples from Peutz-Jegher patients for the presence of apoptotic cells using the TUNEL

staining method together with Hoechst dye staining. Epithelial cells in intestinal villi are in a state of constant turnover [31]. Apoptotic cells are TUNEL positive, and appear as condensed pyknotic nuclear fragments when co-stained by Hoechst dye. In normal non-polypoid intestinal samples, we observed TUNEL positive apoptotic epithelial cells with pyknotic nuclei in almost every villus cross section: 94.9% of villi examined contained one or more TUNEL positive apoptotic cells with pyknotic nuclei (Figure 9A & D). Frequently, the apoptotic epithelial cells appear to be engulfed by other epithelial cells, since they are usually found in the cytoplasm of living epithelial cells (Figure 9A). In contrast, the prevalence of apoptotic cells was much lower in PJS polyps: only 5% of the villi in the polyps contained one or more TUNEL positive apoptotic epithelial cells (Figure 9B & D).

TUNEL-positive apoptotic epithelial cells with pyknotic nuclei are found at normal frequency in the areas of normal intestinal epithelium adjacent to or in between Peutz-Jeghers polyps (Figure 9D), consistent with the normal expression of LKB1 in these cells (Figure 8Ba), providing an excellent internal control for our analysis. Our data showed an excellent correlation between the expression of LKB1 and presence of apoptosis, suggesting that a severe deficiency of epithelial cell apoptosis in the PJS polyps may play a causal role in the benign hamartoma formation.

Although hamartomatous polyps are benign tumors and unlikely to have lost wild type p53 allele, we nevertheless sought to determine the status of p53 in Peutz-Jegher patient intestinal samples as a mutation in p53 might also explain the loss of apoptosis. Using a standard p53 staining procedure [32], we compared the p53 staining of Peutz-Jegher patient samples with that of control. We found that p53 staining is negative in both normal and Peutz-Jegher patient intestinal samples (data not shown), suggesting that Peutz-Jegher polyps most likely maintain wild type p53 expression.

(6) **Key Research Accomplishments**.

Elucidation of a functional role of LKB1 in mediating p53-dependent apoptosis.

(7) **Reportable Outcomes**.

The Peutz-Jegher gene product LKB1 is a mediator of p53-dependent cell death in the small intestine. Philip Karuman, Robert D. Odze, Xun Clare Zhou, Hong Zhu, Tom P. Brien, Christopher D. Bozzuto, Danny Ooi, and Junying Yuan. *Manuscript submitted.*

(8) **Conclusions**.

The small intestine represents one of the most proliferative tissues of the body with cell division occurring approximately every 5 minutes in each crypt. A consequence of such rapid cell division is that about one gram of tissue (10^9 cells) may be produced every 20 minutes or so in humans [31]. Despite its high proliferation rate, the small intestine rarely develops cancer suggesting that this tissue may contain an efficient mechanism for regulating cell proliferation, differentiation and death. The small intestine has a high degree of sensitivity to radiation and chemotherapeutic agents. p53 is potently induced by radiation in stem cells of the small intestine but not in the colon. Thus, in the

small intestine, p53 likely provides an important protective mechanism by eliminating damaged cells before they become neoplastic [33]. Our results suggest that LKB1 may be one of the key molecules in this highly sensitive apoptotic regulatory mechanism.

A dominant negative mutant of LKB1 specifically inhibited the apoptosis induced by disruption of microtubule dynamics, but not that of death receptor activation or disruption of DNA replication, suggesting that LKB1 is a specific, p53-dependent sentinel of microtubule stability. Our data indicate that the mechanism of LKB1 activation during disruption of microtubule dynamics appears to work by the translocation of LKB1 from the cytoplasm to the mitochondria. Consistent with this, we do not observe any changes in LKB1 expression levels in the cells treated by paclitaxel or vincristine (P. Karuman and J. Yuan, data not shown). On the other hand, during development, LKB1 expression is clearly upregulated in dying intestinal epithelial cells. Thus, LKB1 appears to be activated by both a transcriptional dependent and a transcriptional independent mechanism.

The endogenous LKB1 protein is present in both the nucleus and cytoplasm of epithelial cells. However, it appears that only the cytoplasmic LKB1 is recruited into mitochondria during apoptosis. The requirement of p53 for mitochondrial translocation of LKB1 suggests that p53 may regulate an activity which acts to recruit LKB1 to mitochondria. One obvious candidate is the pro-apoptotic protein Bax. However, since Bax-/- EF cells are equally sensitive to LKB1 induced apoptosis as that of wild type EF cells and LKB1 translocation is unaffected (data not shown), Bax is not essential for LKB1 mediated cell death. p53 has also been shown to regulate a number of genes involved in cellular ROS response such as the PIGs [22]. At this point it is not clear which downstream targets of p53 is required for LKB1 translocation and future experiments will address this question.

Although the endogenous LKB1 is translocated to mitochondria in both Fas and paclitaxel induced apoptosis, the dominant negative LKB1 mutant inhibited only paclitaxel, but not Fas, induced apoptosis. This is consistent with the fact that although mitochondria do play a critical role in Fas induced apoptosis in certain cell types, activated caspase-8 may directly activate downstream caspases without amplification through the mitochondrial pathway in many other cell types [34]. Paclitaxel is an effective chemotherapeutic agent [35]that acts as a microtubule stabilizing agent by preventing depolymerization of polymerized tubulin. Since paclitaxel induced apoptosis is at least partially p53- [36] and Bcl-2-dependent [37], the requirement of LKB1 for paclitaxel induced apoptosis is consistent with our hypothesis that p53 regulates a mitochondrial targeting activity required for LKB1 translocation from cytoplasm to mitochondria. Several studies have demonstrated that bcl-2 phosphorylation can be specifically induced by drugs that affect microtubule depolymerization, such as paclitaxel [37,38]. Thus, determining the substrate targets of LKB1 will be of great interest.

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(10) **Appendices.**

Figure legends:

Figure 1. Induction of cell death by LKB1. **A.** Cell death induced by wild type and the kinase domain alone LKB1. Control (GFP alone), GFP fusion expression constructs of mouse LKB1 (LKB1), the LKB1 kinase domain alone (KDA LKB1) with or without Bcl-xL were tranfected into HT1080 cells. Twenty-four hours after transfection, cells were fixed with 4% paraformaldehyde and stained with Hoechst dye. Dead or dying cells with evidence of nuclear morphological changes are indicated by arrows. **B.** Inhibition of LKB1 induced cell death by Bcl-xL and caspase inhibitors. GFP alone control, GFP fusion expression vectors of wild type LKB1 (GFP LKB1), the kinase domain alone mutant (GFP KDA LKB1), the LKB1 ATP binding site mutation K78M (GFP K78M LKB1) and a N-terminal 88-amino-acid deletion of the kinase domain (GFP-del88 LKB1) were transfected into HT1080cells alone or in combination with Bcl-xL, the C287A capase-9 dominant negative (casp9DN), a pan caspase inhibitor zVAD-fmk (100 μM) and the MEK inhibitor PD 98059 (10 μM). The percentage of cell death was quantified by morphological assessment of cells, flat (live) and round (dead) and evidence of nuclear condensation and fragmentation. 2 μg of LKB1 DNA was used and the total amount of DNA normalized to 4 μg of DNA. The results are exhibited as the means ± s.e.m. from at least 3 experiments. **C.** The expression levels of different LKB1 constructs in HT1080 cells and in the presence of zVAD or Bcl-xL as determined by western blotting (a) and a tubulin control western (b). **D.** Activation of caspases in cells expressing LKB1 KDA. HT1080 cells were transfected with expression vectors of GFP-KDA LKB1 (a-c), KDA LKB1 with Bcl-xL (d-f) or KDA LKB1 in the presence of zVAD (g-i). The cells were stained with Hoechst dye (a, d & g), immunostained with CM1 (b, e & h), or viewed for GFP fluorescence (c, f & i). **E.** LKB1 induces cytochrome c release. HT1080 cells were transfected with the expression vectors of GFP-KDA LKB1 in the presence of zVAD (a-f) or K78M LKB1 (g-i). Cells were stained with Hoechst dye (a), immunostained with anti-cytochrome c (b, d, e, g, & h), viewed for GFP (c, f & i), or double imaging for anti-cytochrome c and GFP. d-i are confocal images. Arrowed cells indicate loss of mitochondrial cytochrome c. Small arrows show early colocalization of KDA LKB1 with cytochrome c in the mitochondria. Transfection efficiency of HT1080 cells is about 15%. At least 100 cells were counted for total of three times for each data point.

Figure 2. Phosphorylation of LKB1 on Thr189 as an inhibitory event and identification of a dominant negative LKB1. **A.** Thr189 is phosphorylated. The expression constructs of GFP alone (control), the flag-tagged ATP binding site mutant K78M, the T189A mutant (T189A), the kinase domain alone (KDA) and wild type LKB1 (LKB1) were transfected into HT1080 cells and immunoprecipitations were carried out 12 to 14 hours post-transfection before significant amount of cell death was evident. Immuno-complexes were incubated with in vitro kinase reaction buffer and analyzed by SDS page and autoradiography (top panel). The level of phosphorylation in T189A mutant is significantly reduced compared to the wild type LKB1. The bottom panel is a western blot control for the levels of LKB1 protein expression in the HT1080 cell lysates. **B.** Identification of an activating mutation and a dominant negative mutation. HT1080 cells were transfected with expression constructs of control (GFP alone), GFP-LKB1 (LKB1), the GFP-Kinase domain alone LKB1 (KDA) and GFP T189A LKB1 mutant (T189A) with or without the ATP binding site mutant (K78M) and Bcl-xL. Expression of T189A induced apoptosis as efficiently as the kinase domain alone mutant in HT1080 cells. Co-

expression of the K78M LKB1 mutant inhibited apoptosis induced by T189A but not that by the kinase domain alone (KDA). Coexpression of bcl-xL inhibited apoptosis induced by T189A. Cell death was assessed at 24 hours post-tranfection. The results are exhibited as the means ± s.e.m. from at least 3 experiments. **C.** Peutz-Jegher mutation K78I LKB1 is also a dominant negative mutant. HT1080 cells were transfected with GFP-murine LKB1, GFP-human LKB1 with or without murine K78M LKB1 or human K78I. Twenty-four hours later, apoptosis was determined by morphology. At least 100 cells were counted for three experiments each. The error bars are s.e.m.

Figure 3. LKB1 induced apoptosis requires p53. **A.** LKB1 induced EF cells apoptosis requires p53. Early passage embryo fibroblasts (EF) cells were obtained from an E13.5 day p53-/-, Bax-/- or wild type background and transfected with 1 μg of the indicated GFP construct with or without a wild type p53 expression construct. 1 μg of wild type p53 expressing vector was used to complement the deficiency in p53-/- EF cells. Equivalent early passage cells from a p53-/- and Bax-/- background were also similarly tansfected. Cell death was assayed 24 hours after transfection by morphological assessment. EF cell transfection efficiency is about 5%. **B.** Dominant negative p53 mutant inhibits LKB1 induced apoptosis in HT1080 cells. HT1080 cells infected with control (pBabepuro) virus or p53.175H dominant negative (pWZLHygro p53.175H) virus were transfected with 2 μg of the indicated DNA and cell death assayed at 24 hours post transfection. At least 100 cells were counted for each data point. All results are exhibited as the means ± s.e.m. from at least 3 experiments.

Figure 4. Mitochondrial translocation of LKB1. The full length LKB1 is largely present in both cytoplasm and nucleus while the kinase domain LKB1 is localized in mitochondria. HT 1080 cells were transfected with 2 μg of either GFP-tagged full length LKB1 (GFP-LKB1(a) the inset shows mitochondrial localization of LKB1 when cell death is induced, GFP-tagged LKB1 kinase domain alone (KDA LKB1) (b) or GFP-tagged K78M LKB1 (c). Twenty four hours after transfection, the cells were stained with the mitochondrial specific vital dye Mitotracker, fixed and stained with Hoechst dye. (d). Bcl-xL protects apoptosis induced by KDA LKB1 without altering its mitochondrial localization. HT1080 cells were cotransfected with equivalent amounts of GFP-KDA LKB1 and pcDNA3 Bcl-xL, and stained with Mitotracker and Hoechst dye 24 hours post-tranfection. Bcl-xL protected the nuclear integrity without changing the mitochondrial localization of LKB1.

Figure 5. p53 is required for mitochondrial translocation of LKB1. Control virus (**A**) and p53 dominant negative mutant p53.175H virus (**B**) infected HT1080 cells, wild type (**C**) and p53-/- (**D**) EF cells were transfected with the GFP-KDA LKB1. Cells were costained with Mitotracker and Hoechst dye 24 hours post-transfection to show the mitochondrial recruitment of KDA LKB1 in control virus infected HT1080 cells and in the wild type EF cells, and its nuclear and cytoplasmic localization in the p53 dominant negative virus infected HT1080 cells and p53 -/- EF cells.

Figure 6. Apoptosis induced mitochondrial translocation of LKB1. **A.** Generation of a LKB1 specific rat polyclonal antibody using recombinant His-tagged LKB1.protein. A 55 kD LKB1 protein was specifically recognized by this LKB1 rat polyclonal antibody on

western blot of control HT1080 cells, HT1080 cells that have been subjected to hypoxia treatment for 24 hours or taxol treatment for 16 hours. This polyclonal LKB1 antibody was used to stain control HT 1080 cells (**B**), or HT 1080 cells treated with Fas (7C11) and cycloheximide (1 μg/ml) for 2 hours (**C**) or paclitaxel (100 nM) for 16 hours (**D**). Cells were stained with Mitotracker, then fixed with 4% paraformaldehyde and processed for immunostaining with rat anti-LKB1 polyclonal antibody and goat anti-rat FITC conjugated secondary antibody and followed by Hoechst dye staining. LKB1 is present in both cytoplasm and nuclei of control cells and translocated to mitochondria in cells that have been treated with Fas or paclitaxel.

Figure 7. A dominant negative LKB1 mutant inhibits paclitaxel and vincristine but not that of 5-fluorouracil and doxorubicin. **A.** The expression levels of p21 (a), p53 (b), tubulin (c) in control and p53 175H retrovirus infected cells, and the expression of K78M LKB1 in K78M LKB1 retrovirus infected cells (d). **B.** HT1080 cells were infected with retrovirus expressing K78M LKB1, p53.175H or control virus. Cells were selected for 4-6 days and treated with 5-fluorouracil, doxorubicin, paclitaxel or vincristine at the doses indicated. Apoptosis was determined 24 hrs later by MTT assay. The data were collected from at least 3 data points. The error bars are s.e.m.

Figure 8. The expression of LKB1 in normal and Peutz-Jegher small intestinal samples. **A.** The expression of LKB1 in normal biopsy small intestinal samples. The small intestinal samples were immunostained by a polyclonal rat anti-LKB1 antibody. The expression of LKB1 is higher in the tips of villi (a & b). The LKB1 expression is significantly elevated in the cytoplasm of certain isolated epithelial cells with abnormal nuclei (c & d). White arrows highlight pyknotic cells in the tip of the villus (b). Black arrows highlight the aggregation of LKB1 staining at various stages of cell death as judged by abnormal and pyknotic nuclear morphology (c & d). **B**. The expression of LKB1 in Peutz-Jegher small intestinal samples. The apparent normal region between polyps still immunostained positive for LKB1 (a), while the polyps are negative for LKB1 staining (c & d). b is a control without the primary antibody.

Figure 9. Intestinal epithelial apoptosis in normal and Peutz-Jegher patients. **A.** TUNEL and Hoechst analysis of duodenal and ileal biopsies taken from normal patients. TUNEL-positive apoptotic epithelial cells with pyknotic nuclei as stained by Hoechst dye are marked with arrow heads. **B.** TUNEL and Hoechst analysis of the hamartomatous region of a duodenal polyp from a Peutz-Jegher patient. Note complete a lack of TUNEL-positive epithelial cells in polyps. **C.** TUNEL and Hoechst staining of the normal mucosa region adjacent to the polyp of the same patient. TUNEL-positive apoptotic cells with pyknotic nuclei are marked with arrowheads. **D.** Tabulation of TUNEL positive cells with pyknotic nuclei in small intestinal epithelial cells of normal villi and Peutz Jeghers polyps. Hamartomatous intestinal epithelia centered around submucosa were counted as a "pseudovillus" unit. At least 10 HPF of tissue per polyp were counted and 3 or more 10 μm sections per patient sample were assessed to obtain a representative picture of cell death events throughout the tissue. Apoptotic events in the submucosa and Brunner's glands were not counted.

Figure 1A.

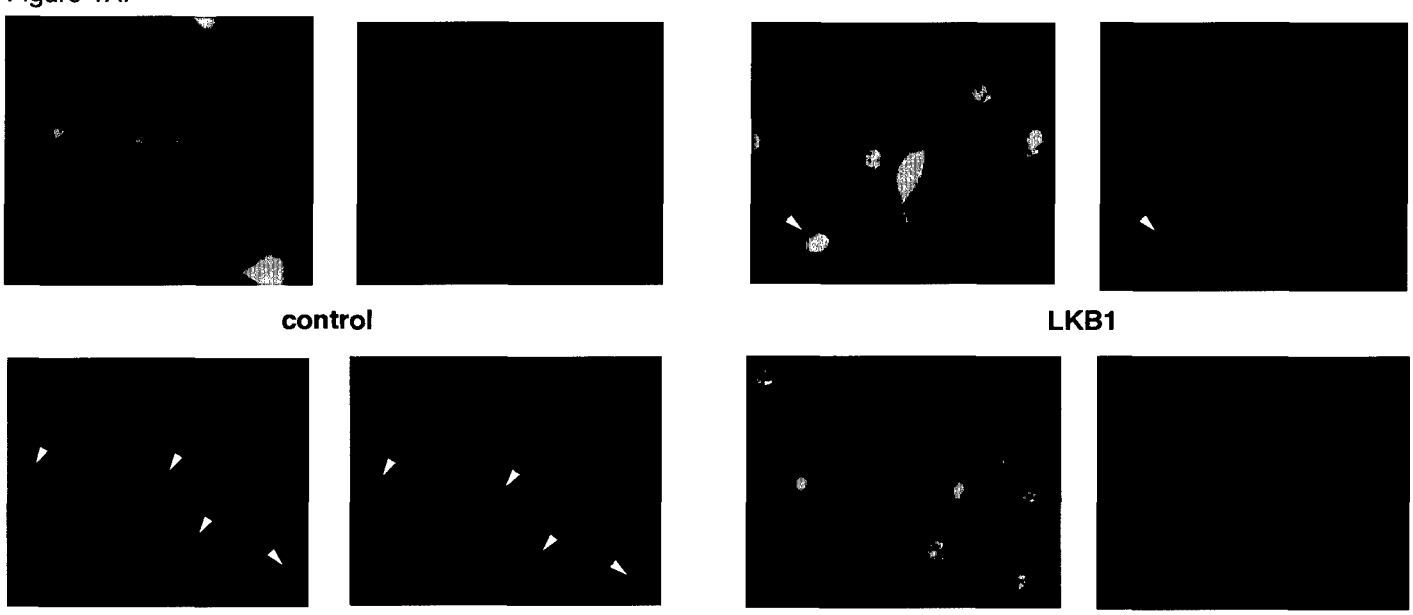


Figure 1B.

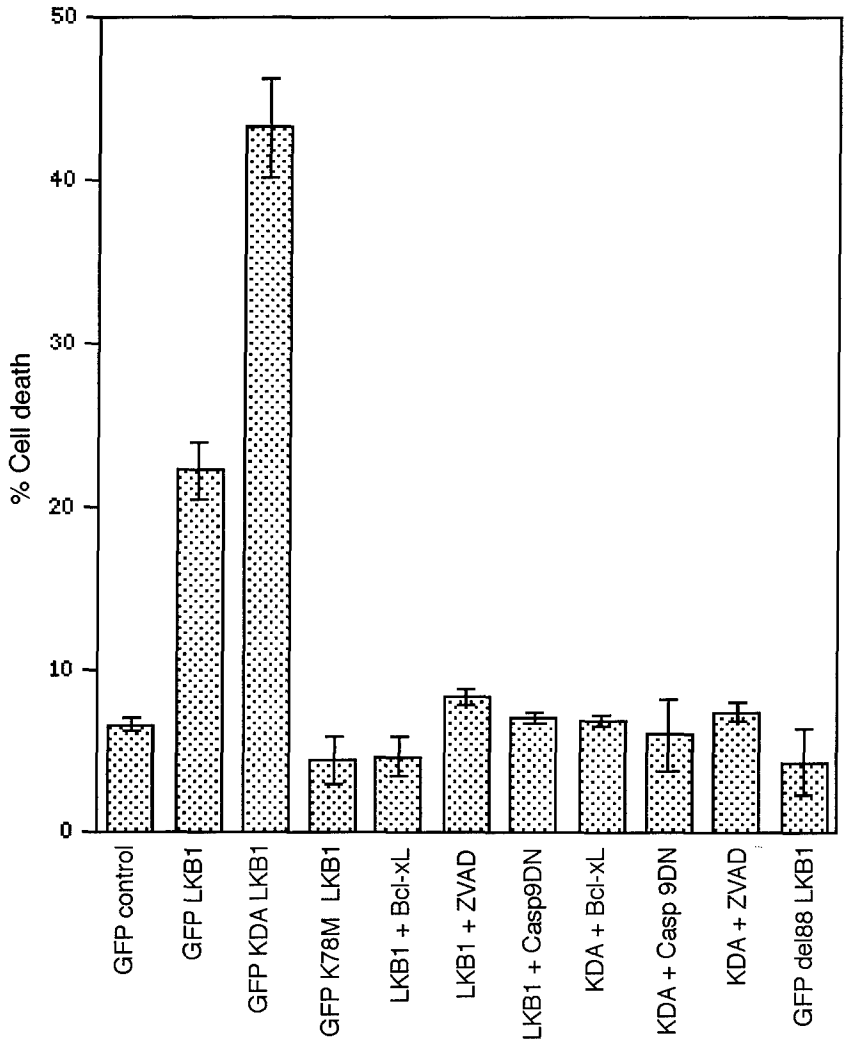

% Cell death
50
40
30
20
10
0
GFP control
GFP LKB1
GFP KDA LKB1
GFP K78M LKB1
LKB1 + Bcl-xL
LKB1 + ZVAD
LKB1 + Casp9DN
KDA + Bcl-xL
KDA + Casp 9DN
KDA + ZVAD
GFP del88 LKB1

Figure 1C.

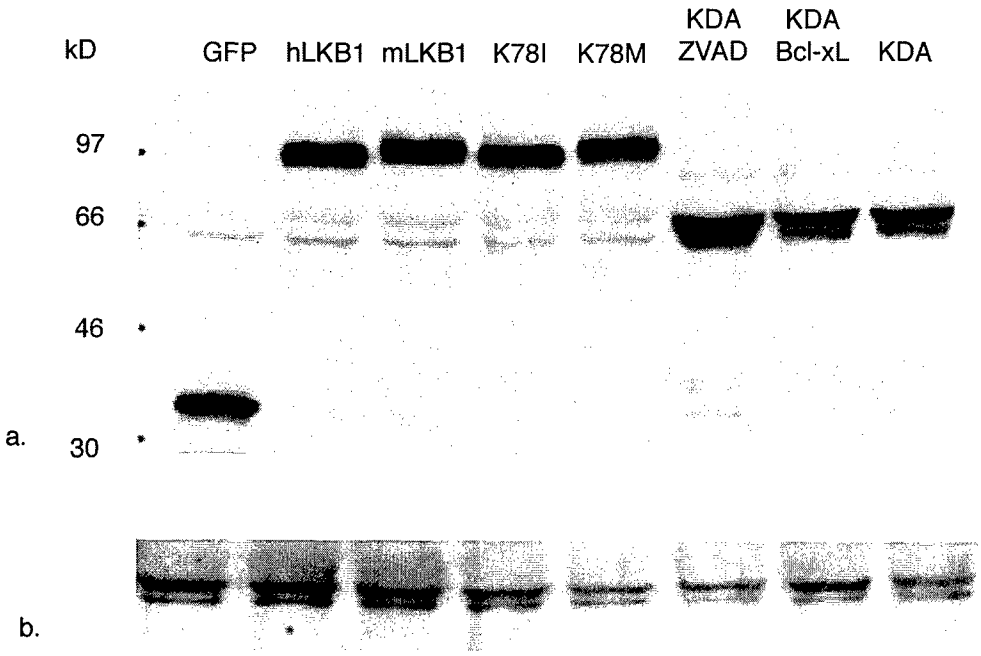

kD
GFP
hLKB1
mLKB1
K78I
K78M
KDA ZVAD
KDA Bcl-xL
KDA
97
66
46
30
a.
b.

Figure 1D.

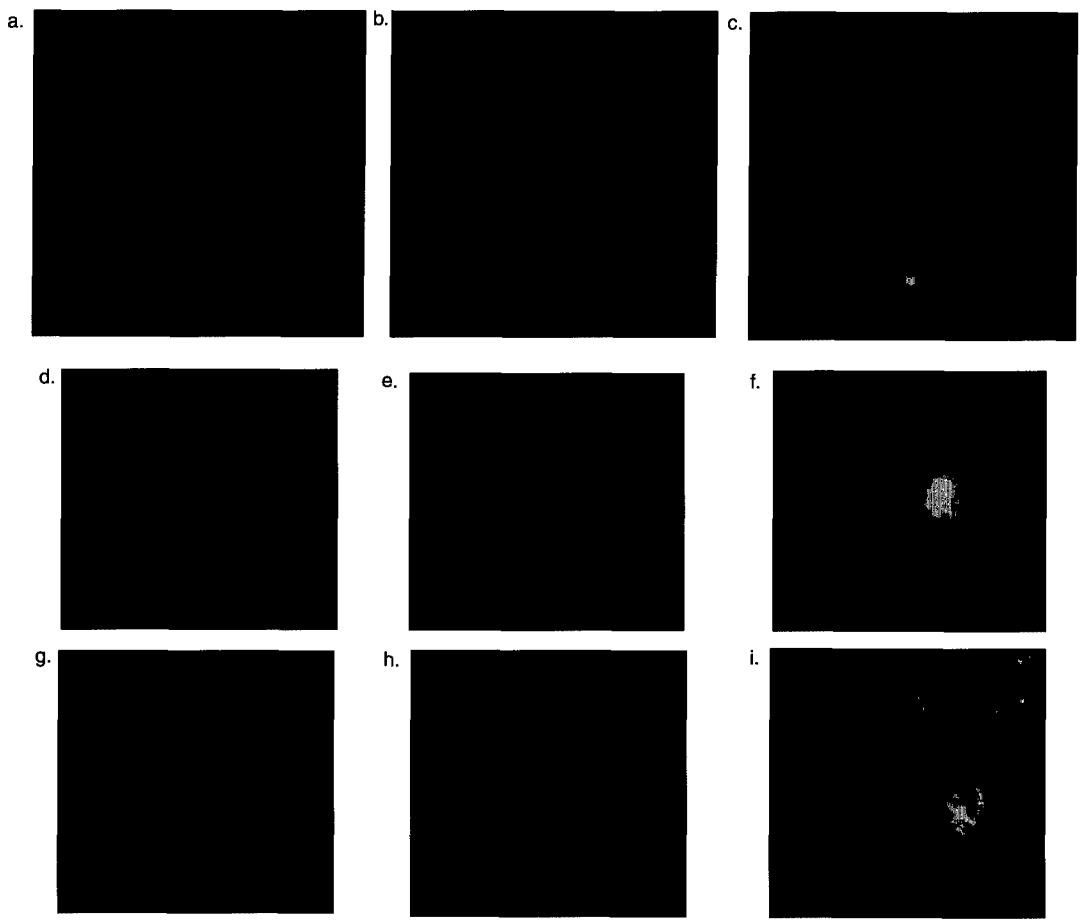
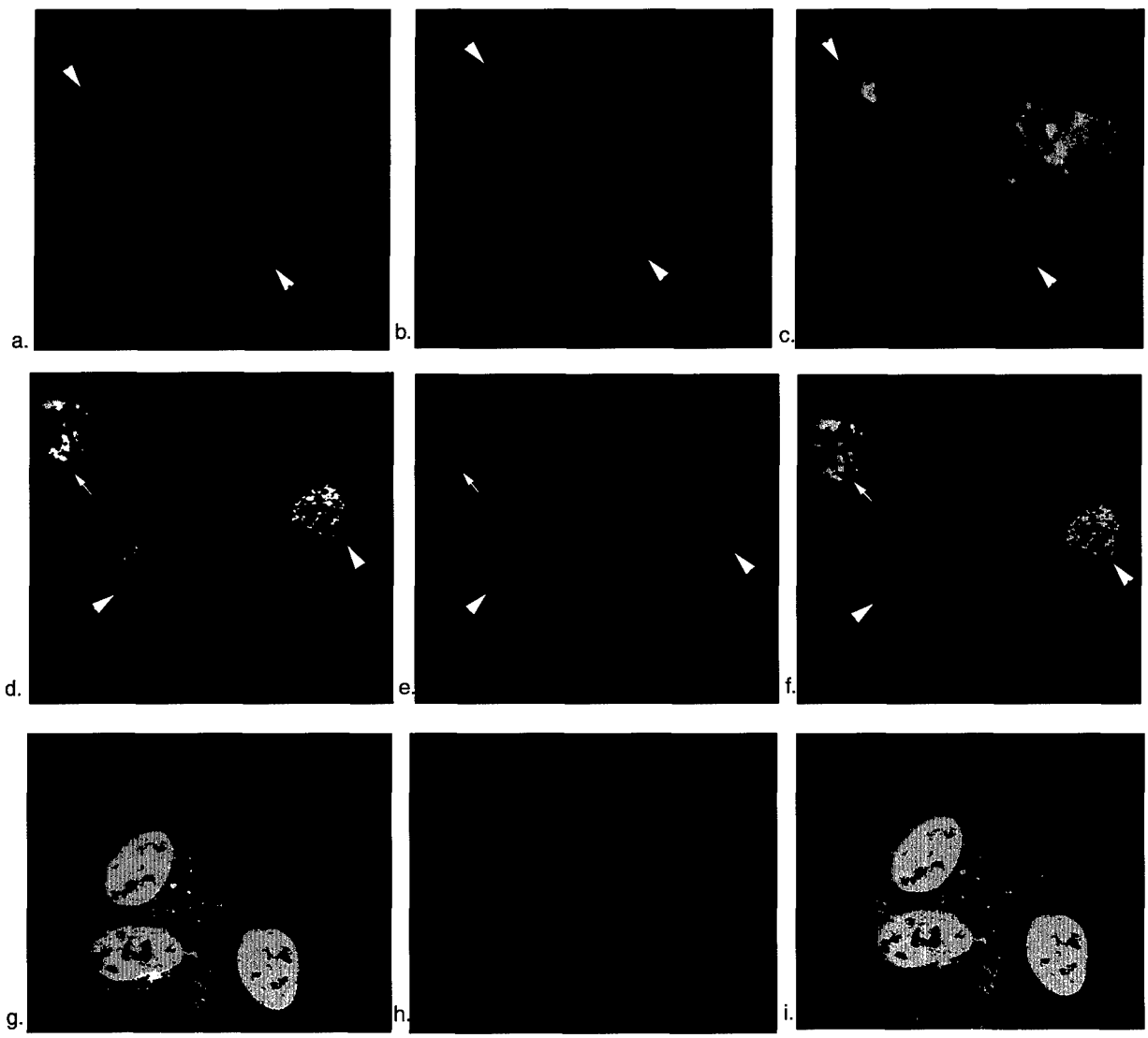


Figure 1E.



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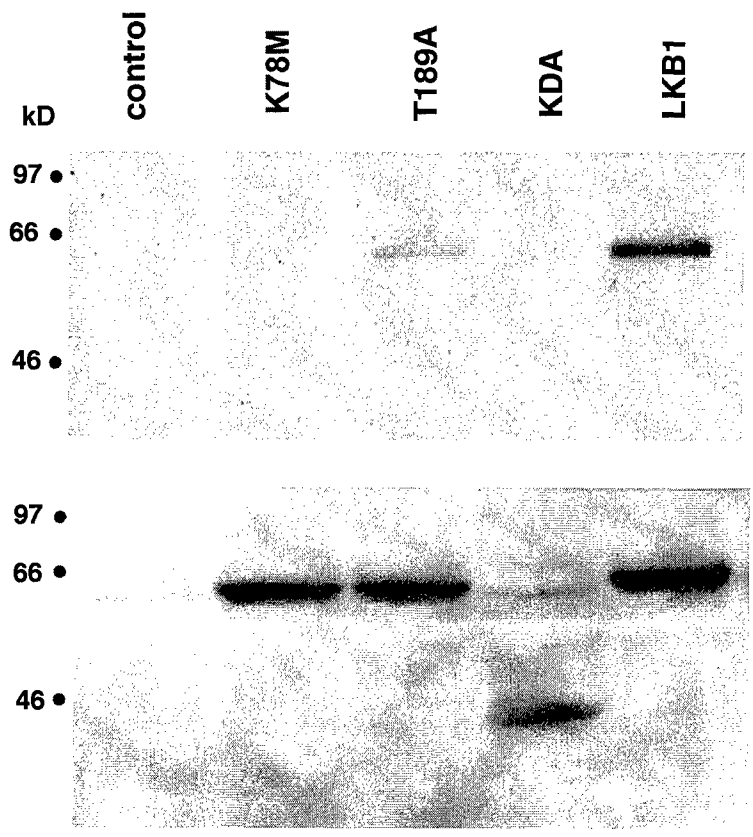


Figure 2B.

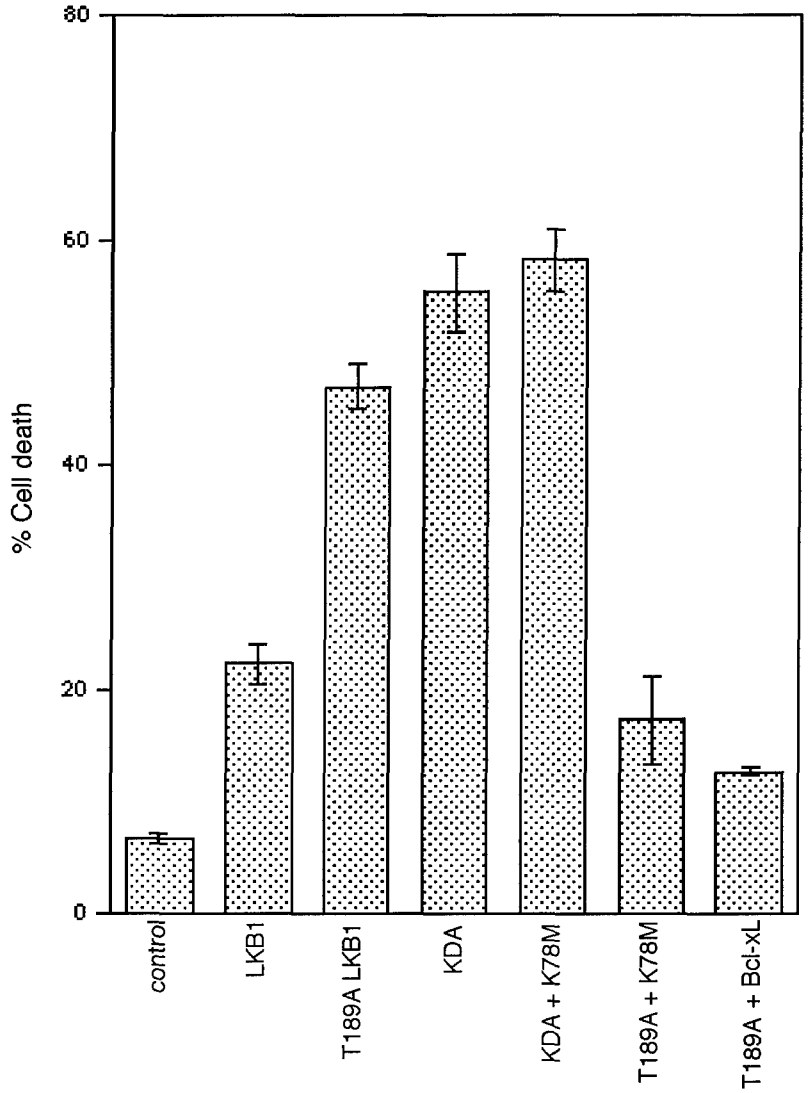

80
60
40
20
0
% Cell death
control
LKB1
T189A LKB1
KDA
KDA + K78M
T189A + K78M
T189A + Bcl-xL

Figure 2C.

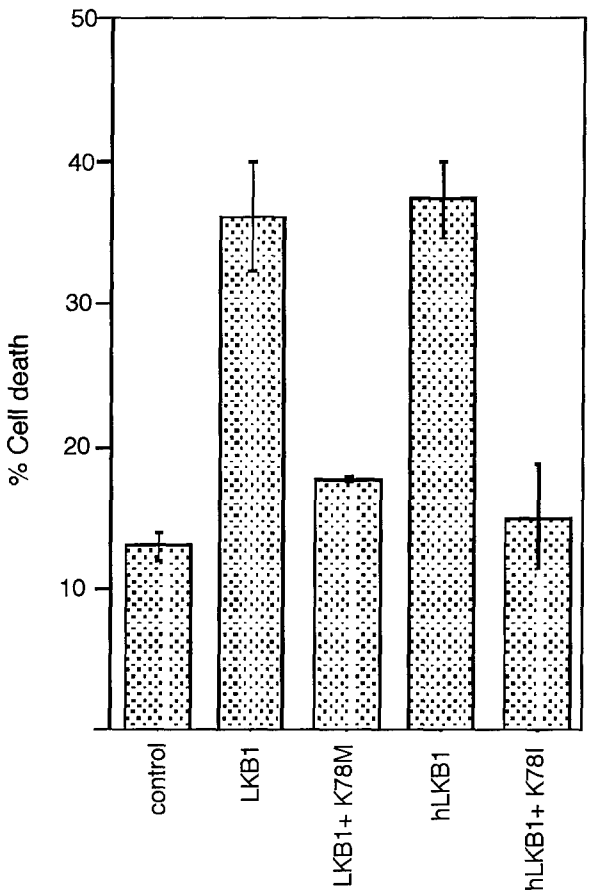

50
40
30
20
10
% Cell death
control
LKB1
LKB1+ K78M
hLKB1
hLKB1+ K78I

Figure 3A.

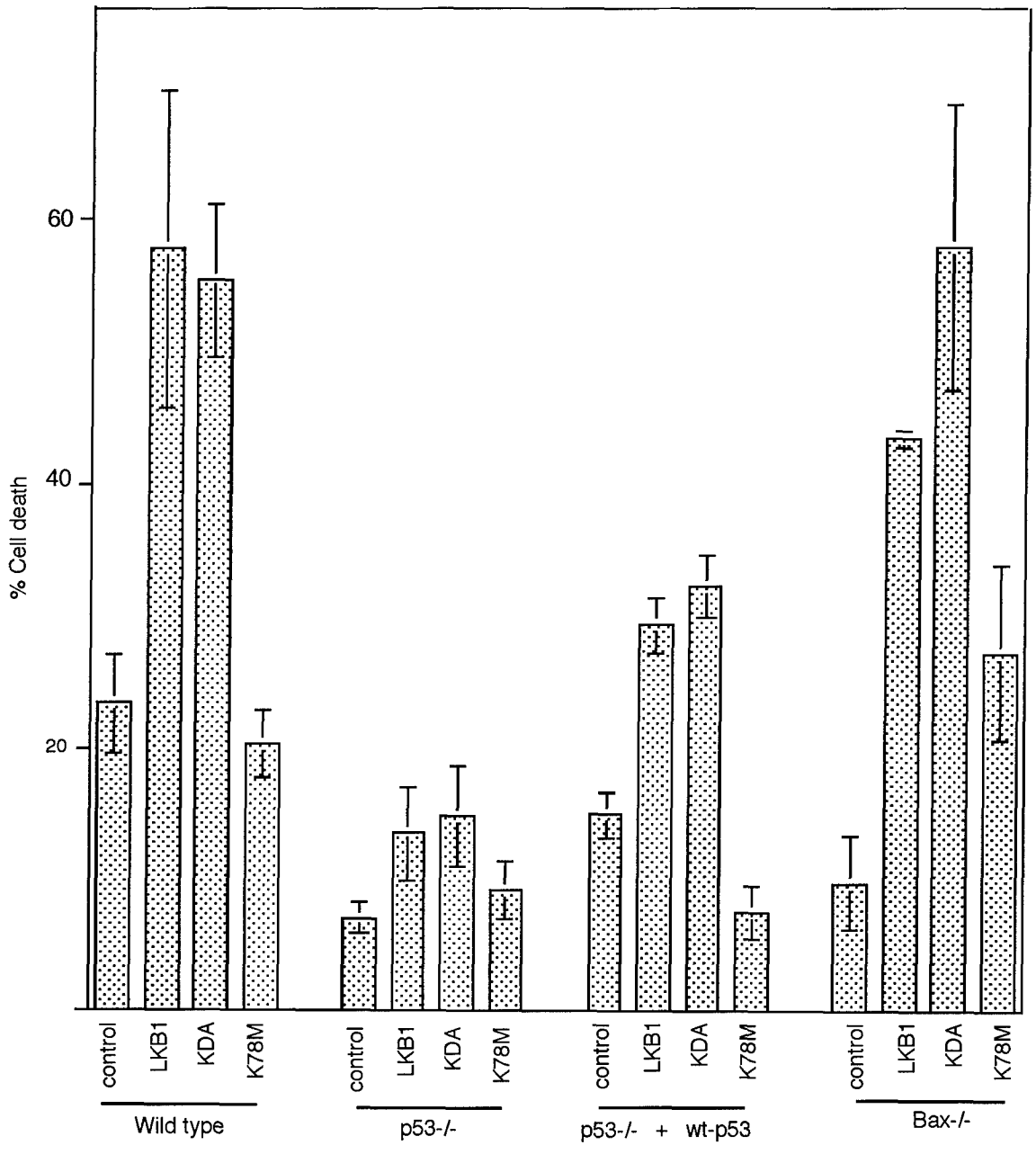

% Cell death
60
40
20
control
LKB1
KDA
K78M
Wild type
p53-/-
p53-/- + wt-p53
Bax-/-

Figure 3B.

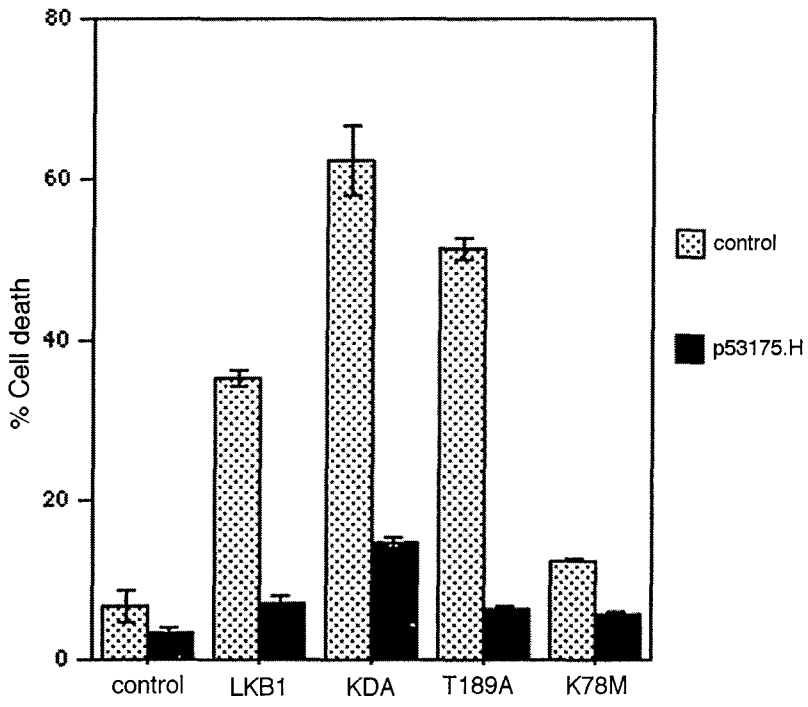

80
60
40
20
0
% Cell death
control
LKB1
KDA
T189A
K78M
control
p53175.H

Figure 4.

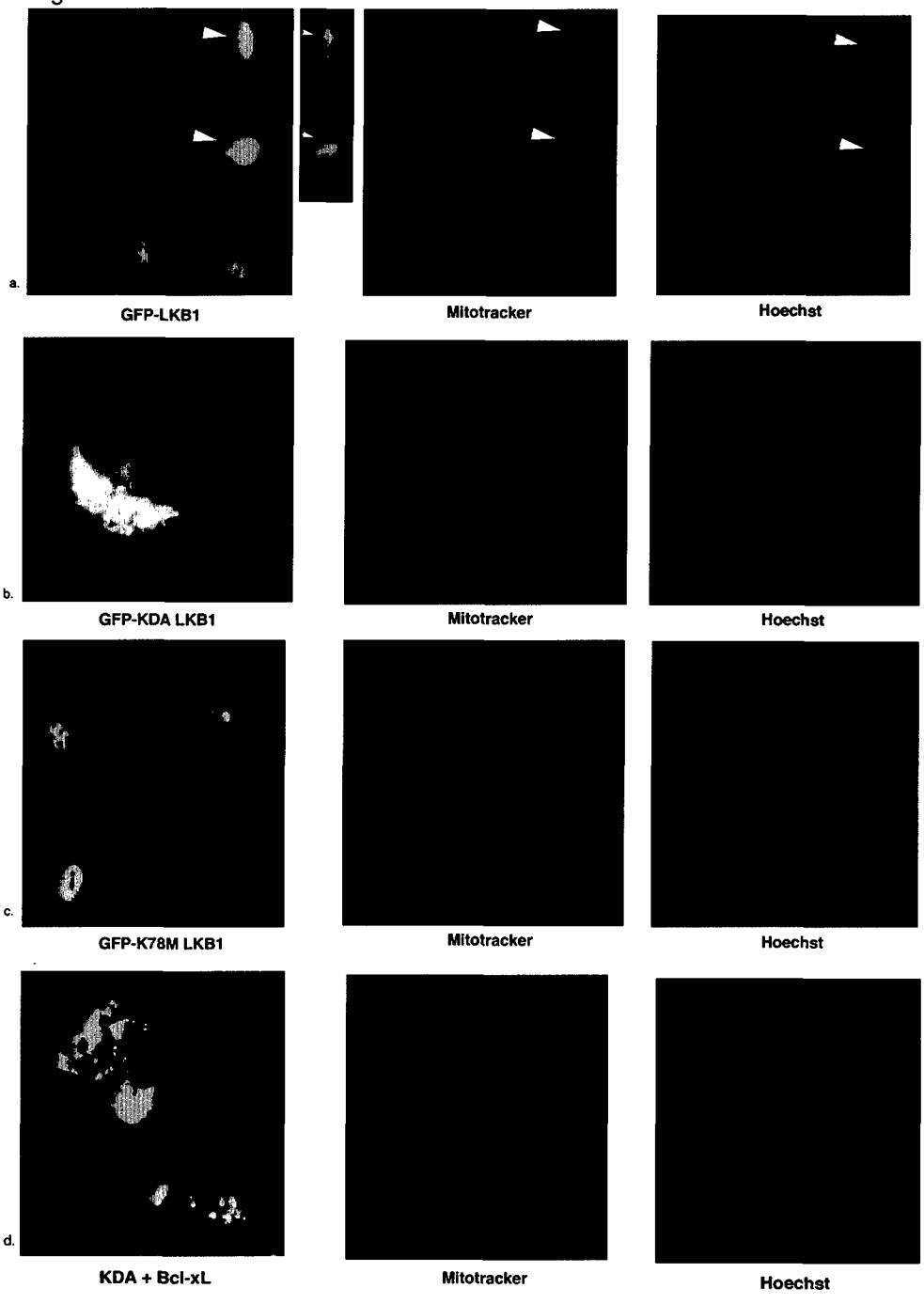
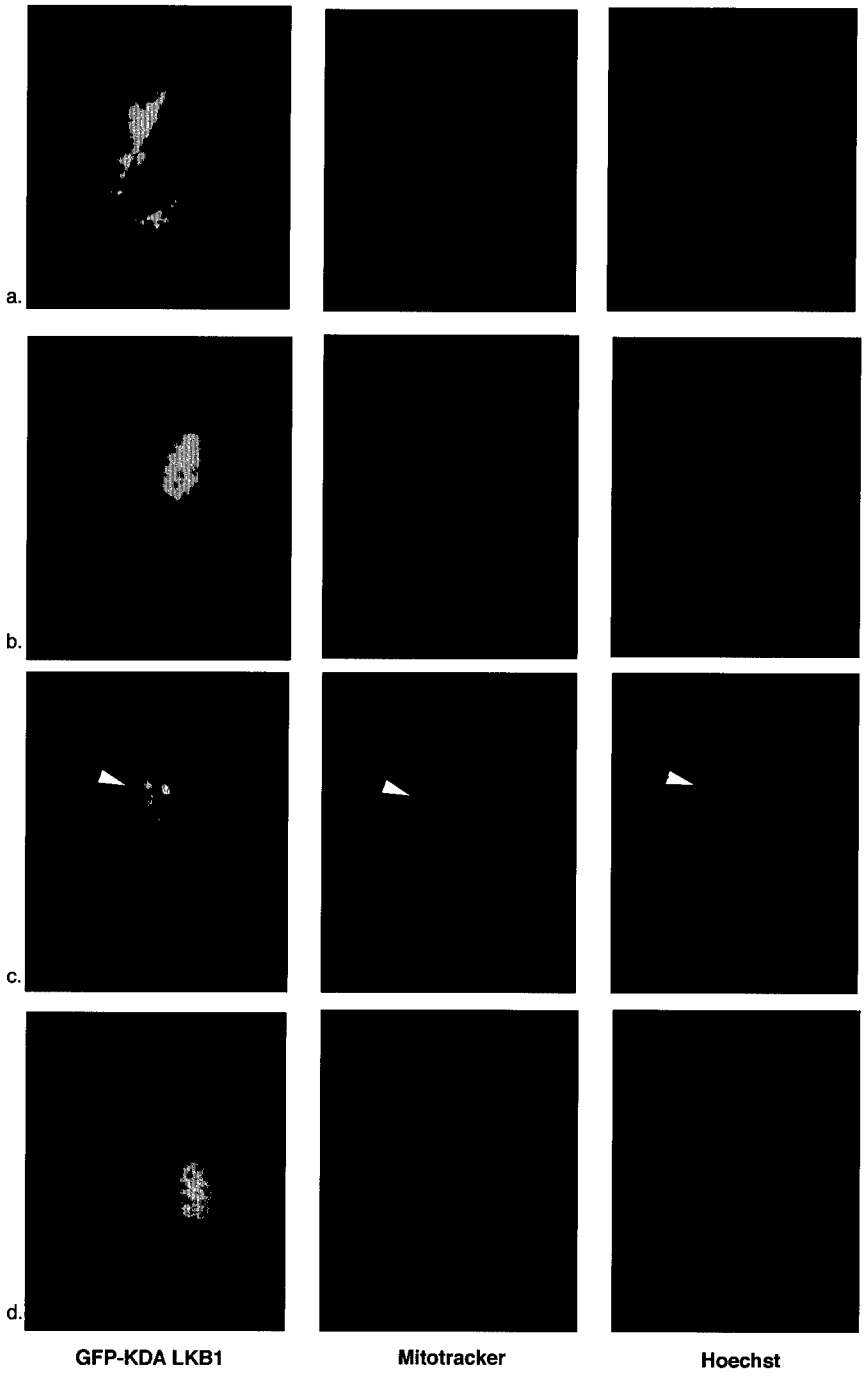

a.
GFP-LKB1
Mitotracker
Hoechst
b.
GFP-KDA LKB1
Mitotracker
Hoechst
c.
GFP-K78M LKB1
Mitotracker
Hoechst
d.
KDA + Bcl-xL
Mitotracker
Hoechst

Figure 5.



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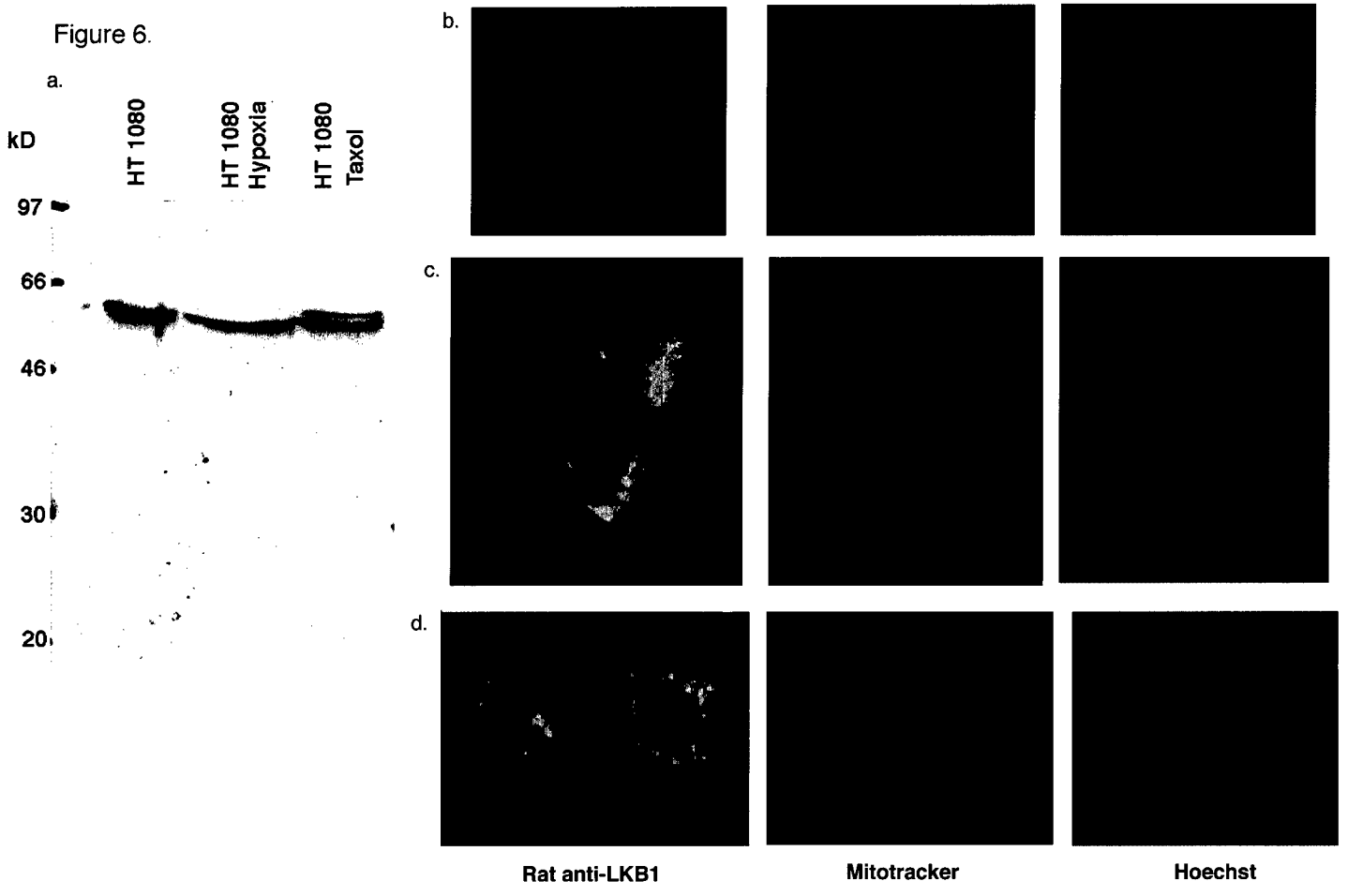

a.
kD
HT 1080
HT 1080 Hypoxia
HT 1080 Taxol
97
66
46
30
20
b.
c.
d.
Rat anti-LKB1
Mitotracker
Hoechst

Figure 7A.

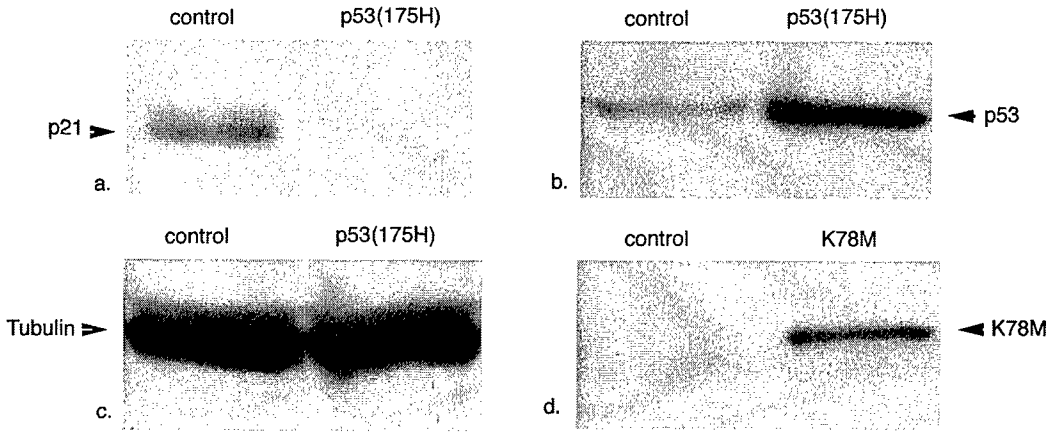

control
p53(175H)
p21
a.
control
p53(175H)
p53
b.
control
p53(175H)
Tubulin
c.
control
K78M
K78M
d.

Figure 7B. 5-Fluorouracil

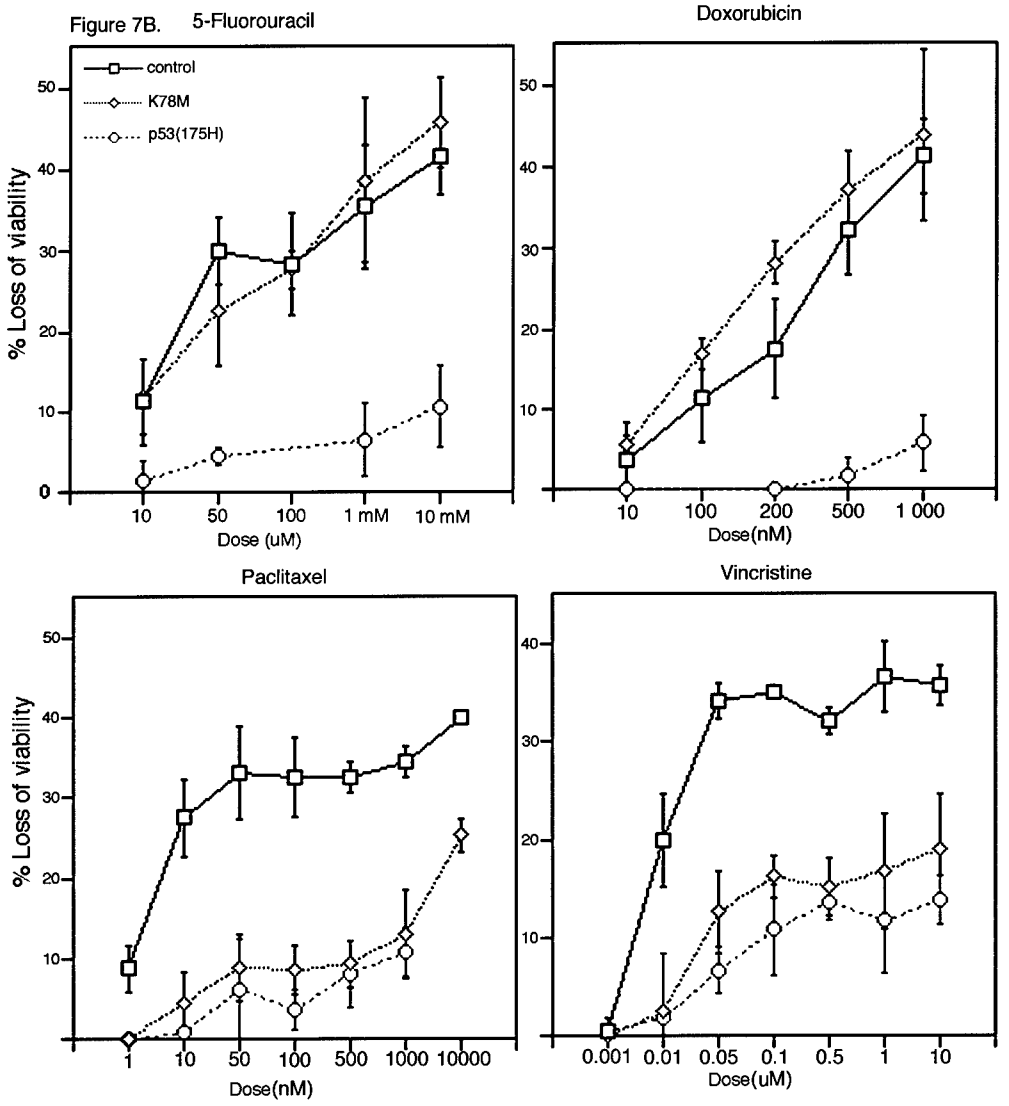


Figure 8A.

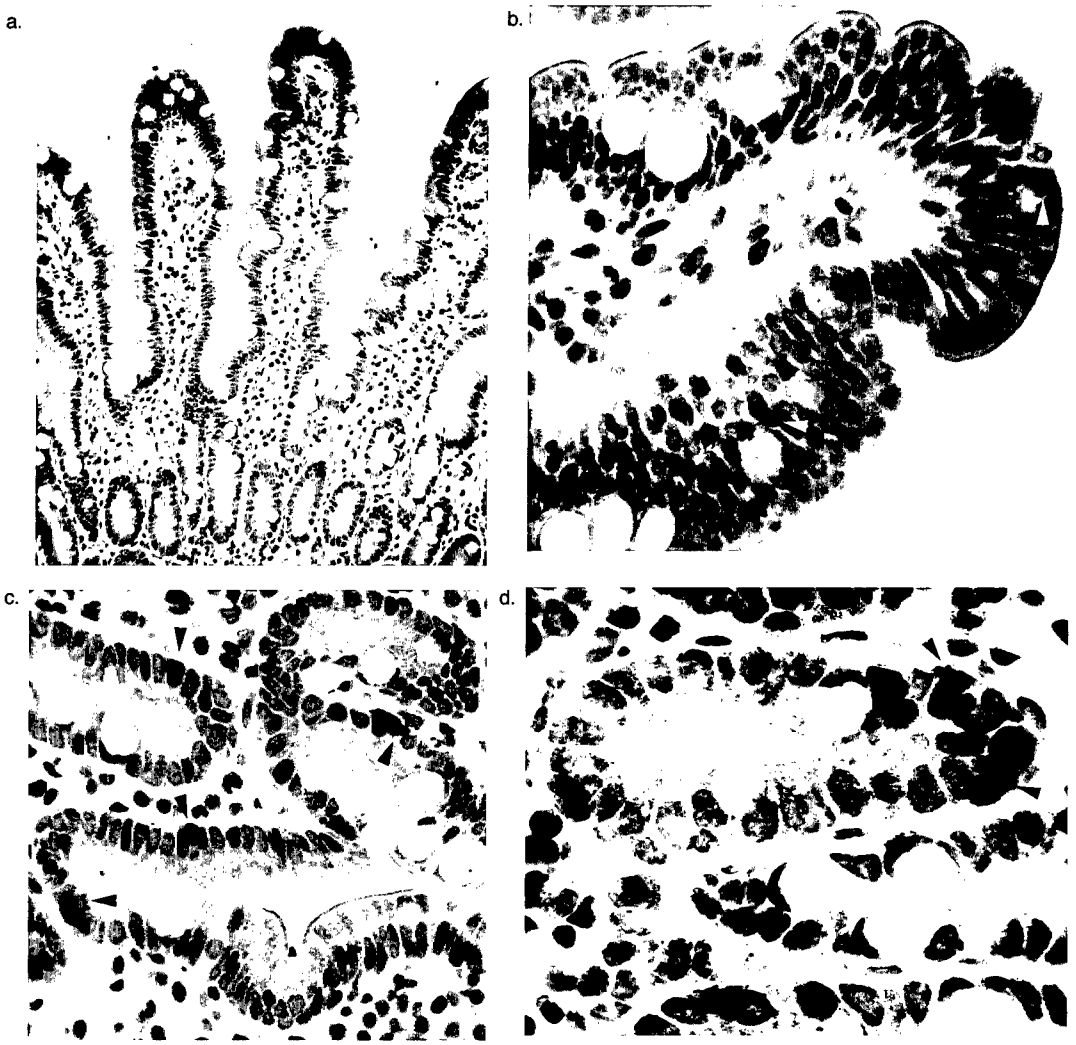


Figure 8B.

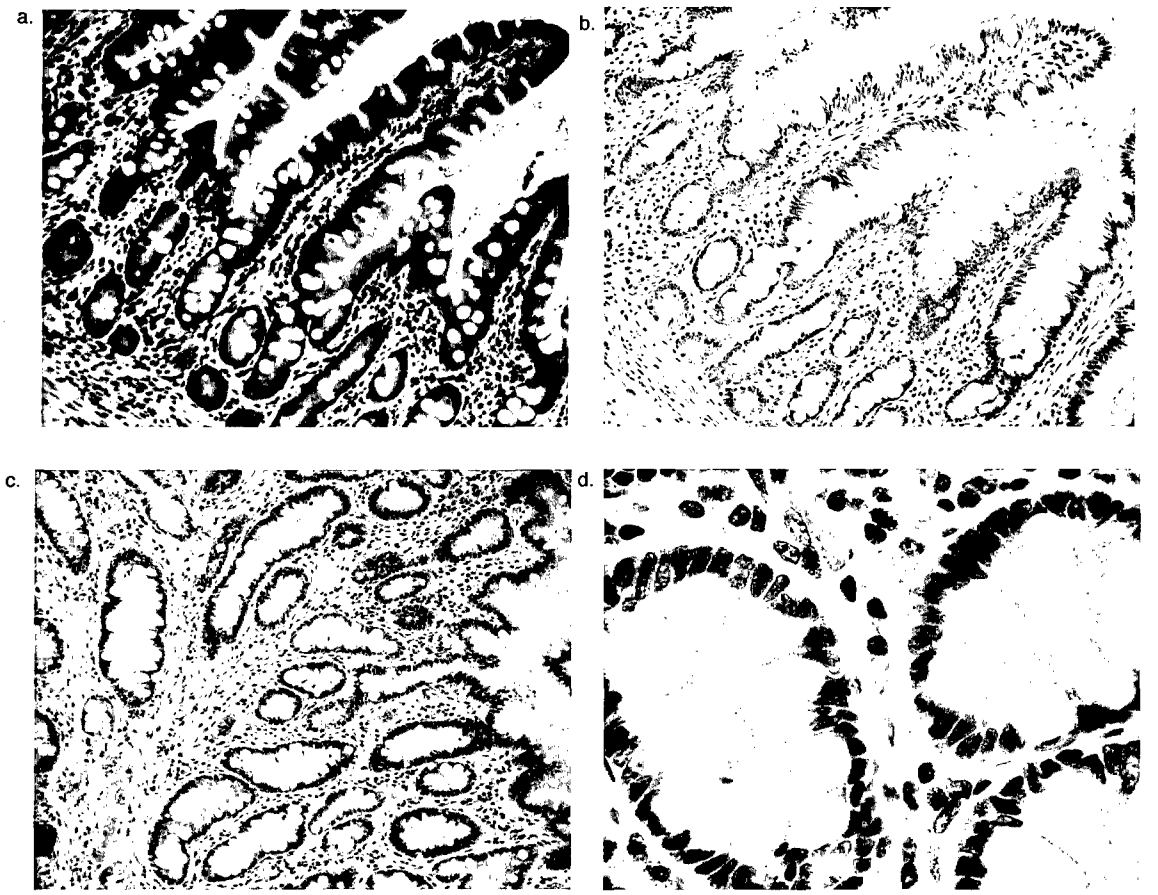
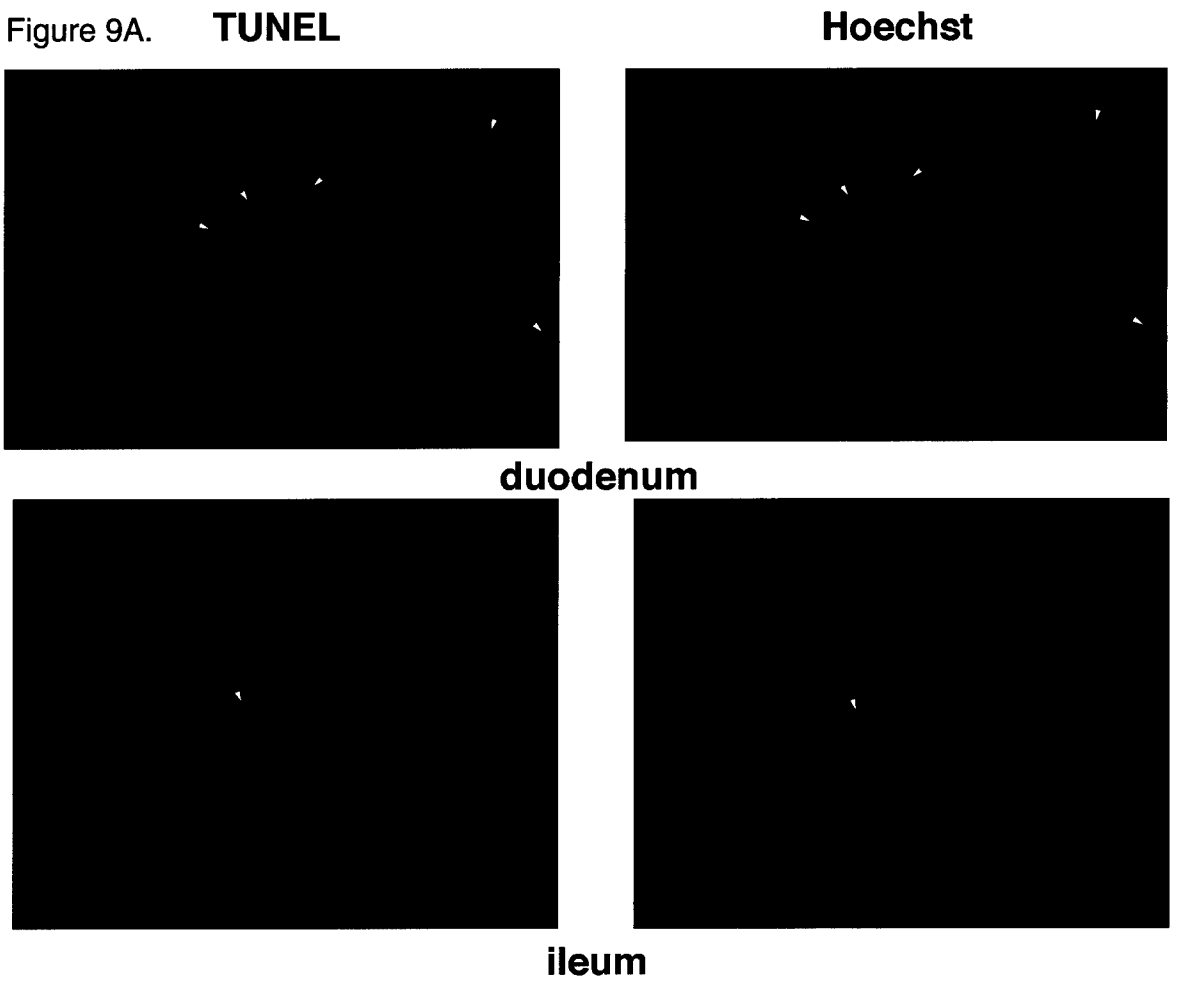


Figure 9A.



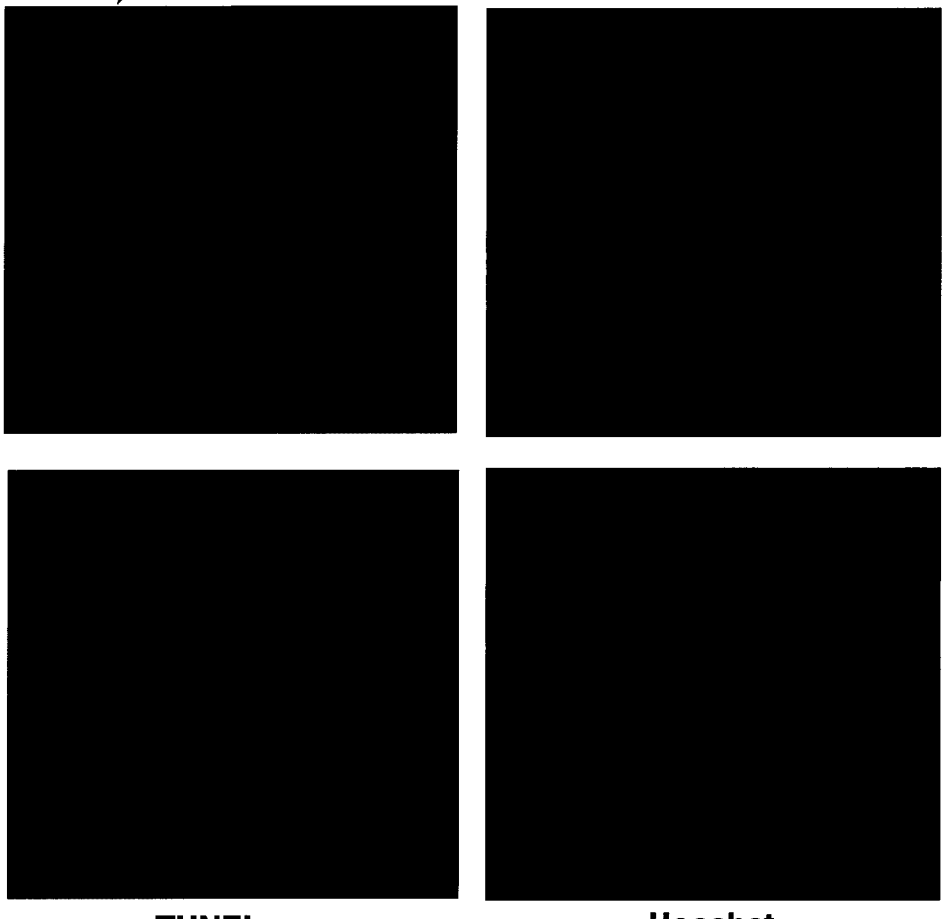


Figure 9B. **TUNEL** **Hoechst**

Figure 9C. **Normal mucosa adjacent to polyp**

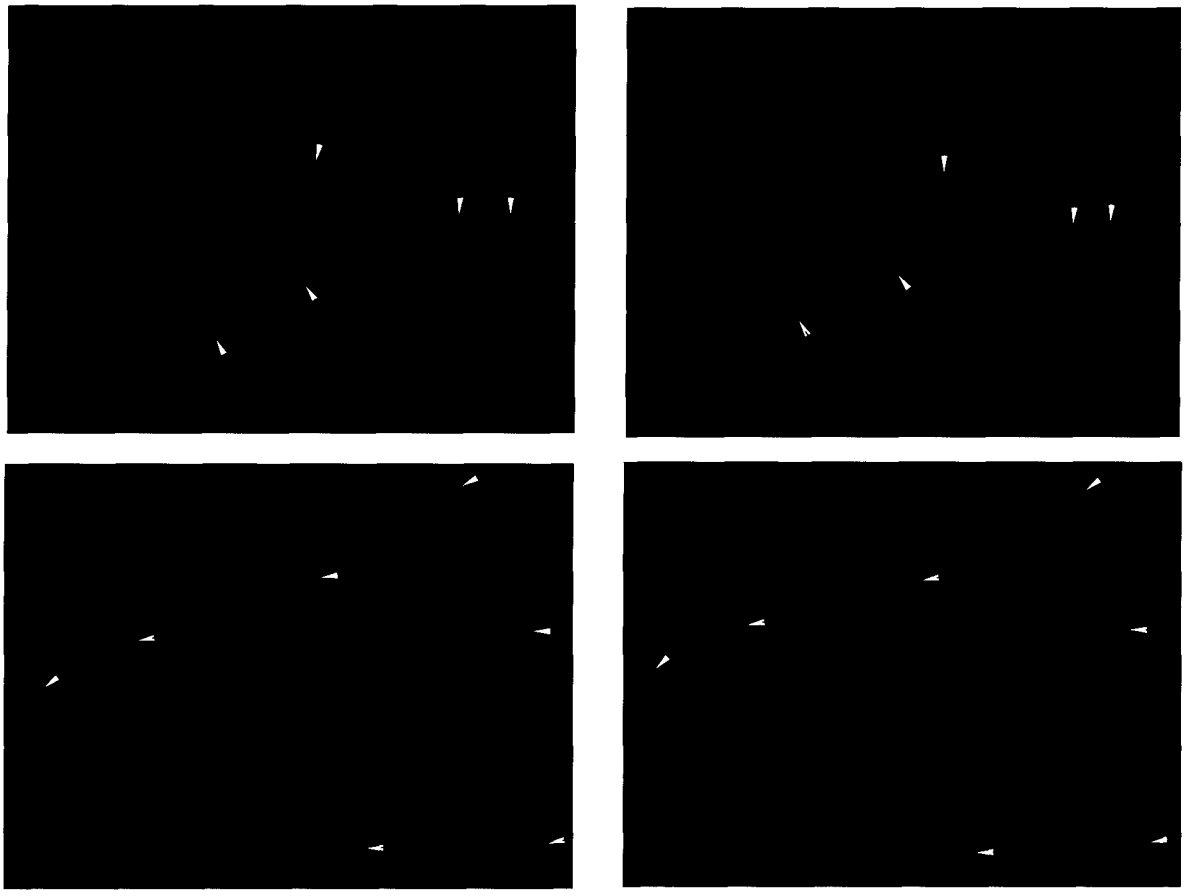


Figure 9D. Apoptotic events in the bowel of Peutz-Jeghers and normal biopsies

	Peutz-Jeghers Polyp (n = 4 patients)		Normal Biopsies (n = 5 patients)	
	# of villus units	# cell death events in villi epithelium	# of villus units	# cell death in villi epithelium
case1.	60	4	40	38
case2.	58	6	38	47
case3.	43	1	40	30
case4.	32	0	9	4
case5.			5	9
Total	193	11	136	129

Mean % of villi with one or more apoptotic events

5.1	94.9

The mean number of apoptotic events in the villi of normal samples compared with the hamartomatous villi is extremely significant. (t-test, $p < 0.0006$)

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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REPLY TO
ATTENTION OF

MCMR-RMI-S (70-1y) 15 May 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
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